

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/00, 15/11, 15/51, A61K 48/00	A1	(11) International Publication Number: WO 95/30746 (43) International Publication Date: 16 November 1995 (16.11.95)
(21) International Application Number: PCT/US95/05812 (22) International Filing Date: 8 May 1995 (08.05.95) (30) Priority Data: 08/240,382 10 May 1994 (10.05.94) US (60) Parent Application or Grant (63) Related by Continuation US 08/240,382 (CON) Filed on 10 May 1994 (10.05.94) (71) Applicant (for all designated States except US): THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WAKITA, Takaji [JP/US]; 666 Main Street, Winchester, MA 01890 (US). WANDS, Jack, R. [US/US]; 210 Varick Road, Waban, MA 02168 (US). (74) Agent: FRASER, Janis, K.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.
(54) Title: ANTISENSE INHIBITION OF HEPATITIS C VIRUS		
(57) Abstract		
The invention features antisense oligonucleotides and methods of using these antisense oligonucleotides for inhibiting HCV RNA translation.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

- 1 -

ANTISENSE INHIBITION OF HEPATITIS C VIRUS

This invention was supported in part by the
5 U.S. Government under grant numbers CA-35711 and AA-08169
awarded by the National Institute of Health. The
Government has certain rights in the invention.

Background of the Invention

This invention relates to antisense inhibition of
10 translation of Hepatitis C Virus RNA.

Antisense oligonucleotides (oligos) are useful
tools for studying cellular and viral gene function
(Uhlmann et al., Chem. Rev. 90(4):543-584, 1990; Stein et
al., Science 261:1004-1012, 1993). In addition,
15 antisense oligos are considered to be ideal agents for
inhibiting viral replication, as they can be specifically
targeted to viral RNA sequences, and therefore are not
likely to affect host-specific gene expression.
Antisense oligos have been used in cell culture systems
20 to inhibit the replication of a number of viruses,
including HIV (Zamecnik et al., Proc. Natl. Acad. Sci.
USA 83:4143-4146, 1986; Matsukura et al., Proc. Natl.
Acad. Sci. USA 84:7706-7710, 1987; Lisziewicz et al.,
Proc. Natl. Acad. Sci. USA 89:11209-11213, 1992),
25 influenza (Kabanov et al., FEBS Lett. 259:327-330, 1990),
herpes simplex (Smith et al., Proc. Natl. Acad. Sci. USA
83:2787-2791, 1986), vesicular stomatitis (Lemaitre et
al., Proc. Natl. Acad. Sci. USA 84:648-652, 1987), and
hepatitis B viruses (Goodarzi et al., J. Gen. Virol.
30 71:3021-3025, 1990; Blum et al., Lancet 337:1230, 1991;
Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992;
Offensperger et al., EMBO J. 12:1257-1262, 1993). The
inhibitory effects of antisense oligos on viral activity
is highly specific. For example, Lisziewicz et al. found
35 that two overlapping 28 nucleotide antisense oligos
inhibited HIV replication, but with a 20-fold difference

- 2 -

in efficiency (Liszewicz et al., Proc. Natl. Acad. Sci. USA 89:11209-11213, 1992).

Hepatitis C virus (HCV) is the major causative agent of post-transfusion hepatitis (Kuo et al., Science 5 244:362-364, 1989). Persistent HCV infection often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Kiyosawa et al., Hepatol. 12:671-675, 1990). Interferon alpha is widely used as an antiviral agent for treatment of chronic HCV infection (Hoofnagle et al., N. 10 Engl. J. Med. 315:1575-1578, 1986; Yoshioka et al., Hepatol. 16:293-299, 1992). However, the antiviral effects of interferon have often been found to be limited and transient.

Summary of the Invention

15 The invention features a method for inhibiting translation of HCV RNA. In this method, HCV RNA is contacted with an oligo that is substantially complementary to a portion of the HCV RNA, and contains a nucleotide sequence selected from the group consisting of 20 SEQ ID NOs: 1-28. An oligo is described herein as being "substantially complementary" or "antisense" to a given nucleic acid, if it is capable of hybridizing to the nucleic acid under physiological conditions. The oligo used in the method of the invention is preferably between 25 about 10 and about 400 nucleotides in length, more preferably is less than about 250 nucleotides in length, more preferably is less than about 100 nucleotides in length, more preferably is less than about 50 nucleotides in length, and most preferably is between about 12 and 30 about 28 nucleotides in length. The oligo can be made of DNA, RNA, or any modifications or combinations thereof that preserve the oligo's ability to hybridize to its complement. For example, the oligo can be an oligodeoxynucleotide, an oligoribonucleotide, a

- 3 -

phosphorothioate oligonucleotide, or a methylphosphonate oligonucleotide, or can contain any combination of these, and other, types of nucleotides and/or inter-nucleotide linkages. A "phosphorothioate oligo" is defined as an
5 oligo containing one or more phosphorothioate inter-nucleotide linkages, while a "methylphosphonate oligo" is defined as an oligo containing one or more methylphosphonate inter-nucleotide linkages. In both of these types of oligos, the modified inter-nucleotide
10 linkages can be present in specific regions of the oligo, e.g., the 5' and/or 3' ends; can be present in random positions within the oligo; or can be present throughout the oligo. It is understood that when the oligo of the invention is a ribonucleotide, "T" in each of the
15 sequences set forth herein represents "U".

In a preferred embodiment, the oligo contains a nucleotide sequence selected from the group consisting of SEQ ID NOs: 2, 6, 7, 14, 16, 17, 20, and 23-26. In another embodiment, the oligo contains a nucleotide
20 sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 8-13, 15, 18, 19, 21, 22, 27, and 28. In another preferred embodiment, the nucleotide sequence of the oligo consists of SEQ ID NO: 2, 6, 7, 14, 16, 17, 20, 23, 24, 25, or 26.

25 The method of the invention can be used to inhibit translation of types I, II, III, IV, and V HCV RNA. The method can be carried out *in vivo*, *ex vivo*, or *in vitro*. The HCV RNA can be in an animal cell, e.g., an hepatocyte, which can be from a mammal, e.g., a human or
30 a chimpanzee. In a preferred embodiment, the oligo and the HCV RNA are contacted with one another in a cell (e.g., a human hepatocyte, or a chimpanzee hepatocyte) by introducing into the cell a vector containing a sequence which is transcribed in the cell to produce the oligo as
35 an oligoribonucleotide.

- 4 -

In another aspect, the invention features a vector containing a nucleotide sequence which is transcribed in an animal cell to generate the oligo of the invention as an oligoribonucleotide. Preferably, the transcribed

5 nucleotide sequence is operably linked to transcription control sequences that function in hepatocytes. Transcription control sequences can include a transcriptional promoter and/or enhancer, and sequences which control the termination of transcription.

10 Transcription control sequences and a transcribed nucleotide sequence are described herein to be "operably linked" if the transcription control sequences control transcription of the transcribed nucleotide sequence. Transcription control sequences that can be used in the

15 vectors of the invention include, but are not limited to, hepatocyte-specific promoters, e.g., the albumin, alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from

20 cytomegalovirus, herpes simplex viruses (types I and II), hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention. A "vector" is defined as a replicable nucleic acid construct. Vectors of the

25 invention include, but are not limited to, plasmid vectors and viral vectors. Preferred viral vectors of the invention include, but are not limited to, those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses, adenovirus, adeno-

30 associated virus, replication defective herpes simplex viruses, and any modified versions of these vectors.

In a final aspect, the invention features a therapeutic composition that can be used in methods of treating or preventing HCV infection. The therapeutic

35 composition contains the oligo of the invention in a

- 5 -

pharmaceutically acceptable carrier, e.g., physiological saline. In a related aspect, the invention features a therapeutic composition containing a vector which contains a nucleotide sequence that is transcribed in an animal cell to generate an oligo of the invention as an oligoribonucleotide, in a pharmaceutically acceptable carrier.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the structure and organization of the 5' region of the HCV genome. The locations of the 4 AUG codons are indicated by downward-pointing arrows. Three small open reading frames (ORFs 1-3) are represented by the boxes containing oblique lines, while the ORF encoding the core protein is indicated by the large open box. The 18S rRNA complementary sequence is located between ORFs 2 and 3 (Brown et al., Nucl. Acids Res. 20:5041-5045, 1992), and is indicated by the black box. Two IRES regions have been reported to be present in the 5' NCR, and are indicated by the smaller open boxes (Tsukiyama-Kohara et al., J. Virol. 66:1476-1483, 1992; Wang et al., J. Virol. 67:3338-3344, 1993). The overlapping oligos of the invention cover the entire 5' NCR and 5' end of the HCV core coding region. The horizontal arrows represent the sense and antisense oligos used in this study. The arrowheads indicate the 3' ends of the oligos.

Fig. 2A is a schematic representation of the structural organization of the vector used to express HCV RNA in this study. A T7 promoter and either HCV type II, or HCV type III, cDNA sequences (nucleotide positions 1 to 1321, or 2 to 1321, respectively) were inserted into

- 6 -

the pUC19 vector. The numbers represent the corresponding nucleotide positions of HCV cDNA. The AUG codon of the HCV core open reading frame is indicated. P22 is a putative HCV nucleocapsid protein, and gp35 is one of the putative HCV envelope proteins (Hijikata et al., Proc. Natl. Acad. Sci. USA 88:5547-5551, 1991).

Fig. 2B is an autoradiogram of the *in vitro* translation products of type II HCV RNA, fractionated by SDS-PAGE. The primary core-envelope protein product migrates at approximately 34 kilo-Daltons (kD), as indicated.

Fig. 3A is a table showing the dose-dependent inhibition of HCV RNA translation by antisense oligo A161 (SEQ ID NO: 6). Sense oligo S120 (SEQ ID NO: 32) was used in this experiment as a negative control. The oligos were added to the reactions at molar ratios varying from 1:1 to 100:1, with respect to template HCV RNA. Percent inhibition of protein synthesis by the two oligos is indicated.

Fig. 3B is a table showing the effects of HCV antisense oligo A161 (SEQ ID NO: 6) and sense oligo S120 (SEQ ID NO: 32) on luciferase mRNA translation.

Fig. 4 is a graph showing the results of fine sequence analysis of antisense oligo effects in the A377 (SEQ ID NO: 17)-defined region of HCV RNA. The numbers after the "A"s indicate the corresponding HCV genomic positions of the 5' ends of the oligos, and the numbers in parentheses indicate the lengths of the oligos. The positions and sequence specificities of the oligos are illustrated on the left. The arrowheads indicate the 3' ends of the oligos. Percent inhibition of HCV type II RNA translation by each of the indicated oligos is shown on the right.

Fig. 5 is an autoradiogram of cDNA synthesis reactions fractionated on a 6% denaturing (urea)

- 7 -

polyacrylamide gel. The reactions were carried out using HCV RNA as the template, and oligos A65 (SEQ ID NO: 2; lane 1), A103 (SEQ ID NO: 4; lane 2), A377 (SEQ ID NO: 17; lane 3), and A387 (SEQ ID NO: 18; lane 4) as primers.

- 5 The predominate cDNA products are indicated by the numbered arrows, and unextended oligo primers are indicated at the bottom of the gel (ODN).

Fig. 6 is an autoradiogram showing the stability of HCV RNA during *in vitro* translation reactions. [α -³³P] 10 labeled HCV RNAs were incubated with antisense oligos A377 (SEQ ID NO: 17; lane 1) and A367 (SEQ ID NO: 16; lane 2), sense oligo S120 (SEQ ID NO: 32; lane 3), and no oligo (lanes 4 and 5). The RNA mixtures were incubated with rabbit reticulocyte lysates (lanes 1-4), and then 15 extracted. Lane 5 shows HCV RNA mixed with rabbit reticulocyte lysate, and extracted immediately, without a 15 minute incubation at 30°C. Full length HCV RNA (1300 nucleotides) is indicated by "a", while the cleaved HCV RNA products of approximately 900 and 380 nucleotides are 20 indicated by "b" and "c", respectively.

Detailed Description

The invention provides antisense oligos that can be used in methods for inhibiting HCV RNA translation. The antisense oligos of the invention can be comprised of 25 DNA, RNA, or any modifications or combinations thereof. As an example of the modifications that the oligos may contain, inter-nucleotide linkages other than phosphodiester bonds, such as phosphorothioate, methylphosphonate, methylphosphodiester, 30 phosphorodithioate, phosphoramidate, phosphotriester, or phosphate ester linkages (Uhlman et al., Chem. Rev. 90(4):544-584, 1990; Anticancer Research 10:1169, 1990), may be present in the oligos, resulting in their increased stability. Oligo stability may also be

- 8 -

increased by incorporating 3'-deoxythymidine or 2'-substituted nucleotides (substituted with, e.g., alkyl groups) into the oligos during synthesis, by providing the oligos as phenylisourea derivatives, or by having
5 other molecules, such as aminoacridine or poly-lysine, linked to the 3' ends of the oligos (see, e.g., Anticancer Research 10:1169-1182, 1990). Modifications of the RNA and/or DNA nucleotides comprising the oligos of the invention may be present throughout the oligo, or
10 in selected regions of the oligo, e.g., the 5' and/or 3' ends. The antisense oligos may also be modified so as to increase their ability to penetrate the target tissue by, e.g., coupling the oligos to lipophilic compounds. The antisense oligos of the invention can be made by any
15 method known in the art, including standard chemical synthesis, ligation of constituent oligos, and transcription of DNA encoding the oligos, as described below.

Hepatocytes are susceptible to HCV infection, and
20 thus are the preferred cellular targets for the antisense oligos of the invention. Targeting of antisense oligos to hepatocytes may be achieved by coupling the oligos to ligands of hepatocyte-specific receptors. For example, the oligos can be coupled to asialo-orosomucoid, (poly)L-
25 lysine-asialo-orosomucoid, or any other ligands of the hepatic asialoglycoprotein receptor (Spiess, Biochemistry 29(43):10009-10018, 1990; Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992; Wu et al., Biotherapy 3:87-95, 1991). Similarly, antisense oligos may be targeted to
30 hepatocytes by being conjugated to monoclonal antibodies that specifically bind to hepatocyte-specific receptors. Antisense oligos may also be targeted to hepatocytes using specific vectors, as described below.

The antisense oligos of the invention may be
35 provided exogenously to a target hepatocyte.

- 9 -

Alternatively, the antisense oligo may be produced within the target cell by transcription of a nucleic acid molecule comprising a promoter sequence operably linked to a sequence encoding the antisense oligo. In this method, the nucleic acid molecule is contained within a non-replicating linear or circular DNA or RNA molecule, is contained within an autonomously replicating plasmid or viral vector, or is integrated into the host genome. Any vector that can transfect a hepatocyte may be used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO89/07136; Rosenberg et al., N. Eng. J. Med. 323(9):570-578, 1990), adenovirus (see, e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993), adeno-associated virus (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sept. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and any modified versions of these vectors. Methods for constructing expression vectors are well known in the art (see, e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New York, 1989).

Appropriate regulatory sequences can be inserted into the vectors of the invention using methods known to those skilled in the art, for example, by homologous recombination (Graham et al., J. Gen. Virol. 36:59-72, 1977), or other appropriate methods (Molecular Cloning: A Laboratory Manual, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New York, 1989). Promoters are inserted into the vectors so that they are operably linked 5' to the nucleic acid

- 10 -

sequence encoding the antisense oligo. Any promoter that is capable of directing initiation of transcription in a eukaryotic cell may be used in the invention. For example, non-tissue specific promoters, such as the

5 cytomegalovirus (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein), mouse metallothionine I gene (Hammer, et al., J. Mol. Appl. Gen. 1:273-288, 1982), HSV thymidine kinase (McKnight, Cell 31:355-365 1982), and SV40 early (Benoist

10 et al., Nature 290:304-310, 1981) promoters may be used. Non-tissue specific promoters may be used in the invention, as expression of antisense HCV oligos in non-liver cells directed by the non-tissue specific promoters should be harmless to the non-liver cells, because of the

15 specificity of the antisense oligos of the invention for viral sequences. However, preferred promoters for use in the invention are hepatocyte-specific promoters, the use of which ensures that the antisense oligos are expressed primarily in hepatocytes. Preferred hepatocyte-specific

20 promoters include, but are not limited to the albumin, alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from cytomegalovirus, herpes simplex viruses (types I and II),

25 hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention.

The antisense oligos of the invention, and the recombinant vectors containing nucleic acid sequences

30 encoding them, may be used in therapeutic compositions for preventing or treating HCV infection. The therapeutic applications of antisense oligos in general are described, e.g., in the following review articles: LeDoan et al., Bull. Cancer 76:849-852, 1989; Dolnick,

35 Biochem. Pharmacol. 40:671-675, 1990; Crooke, Annu. Rev.

- 11 -

Pharmacol. Toxicol. 32, 329-376, 1992. The therapeutic compositions of the invention may be used alone or in admixture, or in chemical combination, with one or more materials, including other antisense oligos or
5 recombinant vectors, materials that increase the biological stability of the oligos or the recombinant vectors, or materials that increase the ability of the therapeutic compositions to penetrate hepatocytes selectively. The therapeutic compositions of the
10 invention may be administered in pharmaceutically acceptable carriers (e.g., physiological saline), which are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers, as well as
15 pharmaceutical necessities for use in pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field, and in the USP/NF.

The therapeutic compositions of the invention can
20 be administered in dosages determined to be appropriate by one skilled in the art. An appropriate dosage is one which effects a reduction in the disease caused by HCV infection, and/or one which is effective at preventing HCV infection. It is expected that the dosages will
25 vary, depending upon the pharmacokinetic and pharmacodynamic characteristics of the particular agent, and its mode and route of administration, as well as the age, weight, and health (including renal and hepatic function) of the recipient; the nature and extent of the
30 disease; the frequency and duration of the treatment; the type of, if any, concurrent therapy; and the desired effect. It is expected that a useful dosage contains between about 0.1 to 100 mg of active ingredient per kilogram of body weight. Ordinarily, 0.5 to 50 mg, and
35 preferably, 1 to 10 mg of active ingredient per kilogram

- 12 -

of body weight per day given in divided doses, or in sustained release form, is appropriate.

The therapeutic compositions of the invention may be administered to a patient by any appropriate mode, e.g., parenterally, intraperitoneally, or intravenously, as determined by one skilled in the art. Alternatively, it may be necessary to administer the treatment surgically to the target tissue. The treatments of the invention may be repeated as needed, as determined by one skilled in the art.

The invention also includes any other methods which accomplish *in vivo* transfer of nucleic acids into eukaryotic cells. For example, the nucleic acids may be packaged into liposomes, non-viral nucleic acid-based vectors, erythrocyte ghosts, or microspheres (microparticles; see, e.g., U.S. Patent No. 4,789,734; U.S. Patent No. 4,925,673; U.S. Patent No. 3,625,214; Gregoriadis, *Drug Carriers in Biology and Medicine*, pp. 287-341 (Academic Press, 1979)). Further, delivery of antisense oligos may be accomplished by direct injection of the oligos into target tissues, for example, in a calcium phosphate precipitate or coupled with lipids.

Experimental Data

MATERIALS AND METHODS

Construction and Expression of HCV cDNAs

HCV cDNAs (nucleotide positions 1 to 1321) were isolated from anti-HCV positive sera taken from two Japanese individuals (TH and NT; kindly provided by Dr. Shinichi Kakumu, Nagoya University, Japan) using the polymerase chain reaction (PCR), as described (Yoshioka et al., *Hepatology* 16:293-299, 1992). Amplified cDNA fragments were inserted into the pUC19 cloning vector. The nucleotide sequences of 3 clones obtained from each individual were determined by the di-deoxy chain termination sequencing method. The clones exhibited

- 13 -

0.3 to 0.6% nucleotide sequence variation within the same individual, and 21.6 to 21.9% nucleotide sequence variation between the two individuals. Clones from TH exhibited 94.2 to 95.1% nucleotide sequence homology to
5 HCV-BK (Takamizawa et al., J. Virol. 65:1105-1113, 1991) and HCV-J (Kato et al., Proc. Natl. Acad. Sci. USA 87:9524-9528, 1990), which are type II HCV clones. One clone from TH, designated 2-2, was selected for further analysis. Clones from NT exhibited 94.2 to 94.5%
10 nucleotide sequence homology to HCV-J6, which is a type III HCV clone (Okamoto et al., J. Gen. Virol. 72:2697-2704, 1991). A clone designated 4-1 was selected from patient NT for further study. Bacteriophage T7 promoter sequences were inserted upstream of the 5' ends of the
15 HCV inserts (Fig. 2), and the resulting expression vectors, designated pTH for the type II HCV genotype, and pNT for the type III HCV genotype, were used for further experiments.

Design and synthesis of oligos

20 A series of sense and antisense oligodeoxynucleotides were synthesized based on the sequence of pTH type II HCV by standard phosphoramidite chemistry using a Milligen/Biosearch 8750 synthesizer. The oligos were purified after NH_4OH detachment 55°C (6
25 hours) and NAP 25 column (Pharmacia, Piscataway, NJ) desalting with 0.1 M NaHCO_3 by reverse phase HPLC (Tritylon, TEAA 0.1 M pH 7.25) and an acetonitrile gradient. The oligos were lyophilized, de-blocked with 1
30 M acetic acid for 1 hour, neutralized with TEA, passed through a NAP 10 column, and lyophilized to dryness. The HCV nucleotide positions to which the oligos used in this study correspond are shown in Fig. 1. In combination, the antisense oligos are complementary to the entire HCV
35 5' NCR, and part of the HCV core region. The number given to each oligo indicates the position on the HCV

- 14 -

genomic map to which the 5' end of the oligo corresponds. The numbers in parentheses, as shown in Fig. 4, indicate the lengths of the oligos. The sizes and G-C contents of the oligos are shown in Table I. The molar

5 concentrations of the oligos used in the experiments were adjusted according to sequence lengths.

***In vitro* transcription and translation of HCV RNA**

pTH and pNT were linearized using *Hind*III, digested with proteinase K, extracted with
10 phenol/chloroform, and precipitated in ethanol, prior to *in vitro* transcription. Transcription reactions were carried out using T7 RNA polymerase (Promega, Madison, WI), and the resulting transcripts were purified by urea-polyacrylamide gel electrophoresis (urea-PAGE), followed
15 by precipitation in ethanol. RNA transcripts were labeled with [α -³³P]UTP (New England Nuclear, Billerica, MA) during the transcription reactions, as required. Type II HCV RNA was used in this study, unless otherwise indicated.

20 One μ g of HCV RNA was mixed with a 10-fold molar excess of sense or antisense oligo, or with distilled water. The mixtures were heated at 70°C for 5 minutes, and then at 50°C for 10 minutes, followed by incubation at room temperature for 10 minutes, in order to complete
25 annealing of target HCV RNA and the oligo. In some experiments, the annealing step was omitted. HCV RNA incubated in the absence of oligo, and rabbit reticulocyte lysate incubated without HCV RNA, were used as controls. *In vitro* translation reactions were carried
30 out as described (Pelham et al., Eur. J. Biochem. 67:247-256, 1976), with minor modifications. Rabbit reticulocyte lysates (Promega, Madison, WI) were added to HCV RNA, or HCV RNA-oligo hybrids, to a final volume of 25 μ l in the presence of [³⁵S]-methionine (New England
35 Nuclear, Billerica, MA). After incubation at 30°C for 1

- 15 -

hour, 4 μ l of each of the reaction mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were dried, and exposed to X-ray film, and the developed films were
5 analyzed by densitometric scanning.

Two μ l of each reaction mixture were precipitated with TCA. The specific incorporation of radioactivity into the translated protein products was determined using a liquid scintillation counter (Pelham et al., Eur. J.
10 Biochem. 67:247-256, 1976). Typically, between 500-5000 cpm/ μ l were detected in the translated protein products. Background counts were around 500 cpm/ μ l in the control mixture containing no HCV RNA. The amounts of HCV core envelope fusion protein produced in control
15 reactions were used as standards, and the amounts of HCV protein synthesized in the presence of the oligos were compared after subtraction of background counts. The percent inhibition of HCV RNA translation by each oligo was calculated using this data.

20 **cDNA synthesis from HCV RNA**

Oligos A65, A103, A377, and A387 (SEQ ID NOs: 2, 4, 17, and 18, respectively) were end-labeled with [γ^{33} P]-ATP (New England Nuclear, Billerica, MA) using T4 polynucleotide kinase (Promega, Madison, WI). Specific
25 incorporation of radioactivity into the oligo was determined after purification through a Sephadex G-25 spin column (Boehringer Mannheim, Germany). The specific activity of each oligo was normalized by adding an appropriate amount of unlabeled oligo. The labeled
30 oligos were hybridized with HCV RNA under the same conditions as described above for the *in vitro* translation reactions, and the cDNAs were synthesized by adding Moloney Murine Leukemia Virus reverse transcriptase (Gibco BRL, Gaithersburg, Maryland). An
35 aliquot of each reaction mixture was fractionated by

- 16 -

denaturing urea-PAGE. The gel was dried, and the products were visualized using autoradiography.

HCV RNA stability

Oligos A377 (SEQ ID NO: 17) and A387 (SEQ ID NO: 18) were hybridized with [α -³³P]-labeled HCV RNA, incubated under the same conditions as described above, and added to rabbit reticulocyte lysate in order to determine the level of RNaseII activity present in the *in vitro* translation reactions. After an incubation at 30°C for 15 minutes, the RNA was extracted (Chomczynski et al., Anal. Biochem. 162:156-159, 1987), and aliquots of the extracted RNA were analyzed by denaturing urea-PAGE.

RESULTS

In vitro translation of HCV protein

A cell free *in vitro* translation system was used to express the HCV core envelope fusion protein, and to determine whether antisense oligos are capable of inhibiting HCV RNA translation. The expression vector used in these experiments contains the complete HCV 5' NCR, and a structural coding region containing the entire core and part of the envelope region of type II and type III HCV cDNAs (Fig. 2). The RNA transcribed from this vector contains 980 nucleotides of HCV coding region. The *in vitro* translated product from type II HCV RNA fractionates as a 34 kD protein by electrophoresis (Fig. 2). That the *in vitro* translated protein product is related to HCV, was confirmed by immunoprecipitation with polyclonal anti-HCV antibodies and monoclonal antibodies raised against recombinant HCV core protein.

Inhibition of HCV RNA translation by antisense oligos

A series of antisense and sense oligos corresponding to the HCV 5' NCR were analyzed for their effects on *in vitro* translation of HCV RNA (Fig. 1). These experiments were performed in duplicate, and repeated at least twice for each oligo shown in Fig. 1.

- 17 -

HCV RNA was incubated in a rabbit reticulocyte lysate system after hybridization with each oligo, and the amount of protein produced in these reactions was determined by measuring the levels of radioactivity incorporated into the core envelope fusion protein. Further quantification was carried out by densitometric scanning of exposed X-ray films. Table I summarizes the results of these analyses, showing the effects of each oligo on translation of both type II and type III HCV RNA. Sense oligos either inhibited HCV RNA translation only very weakly, or actually stimulated HCV protein synthesis, compared to reactions lacking oligos. In contrast, most of the antisense oligos inhibited HCV RNA translation to significant levels, compared to reactions lacking oligos. For example, six antisense oligos, A65, A161, A175, A339, A367, and A377 (SEQ ID NOs: 2, 6, 7, 14, 16, and 17, respectively), exhibited more than 50% inhibition of both type II and type III HCV RNA translation. The target sequence of A161 (SEQ ID NO: 6) is in a highly conserved domain in the HCV 5' NCR. However, other antisense oligos that overlap with this region of HCV RNA exhibited significantly less inhibition of HCV RNA translation than A161. For example, A147 (SEQ ID NO: 5) demonstrated only 20.4% inhibition. A367 (SEQ ID NO: 16), which is complementary to the initiation codon and HCV core coding region, showed the highest level of inhibition of HCV RNA translation (97.6%) of the oligos tested. Oligos A367 (SEQ ID NO: 16) and A377 (SEQ ID NO: 17), which overlap with one another over a 17 nucleotide-long region, both showed a substantial inhibition of both type II and type III HCV core protein synthesis, even though the pNT HCV type III RNA has a one nucleotide mutation or mismatch when compared to the A367 (SEQ ID NO: 16) oligo target sequence. However, other overlapping oligos in this region, as illustrated by

- 18 -

oligos A349 (SEQ ID NO: 15) and A387 (SEQ ID NO: 18), demonstrated only minor inhibitory effects, even though A349 (SEQ ID NO: 15) is complementary to the core AUG start codon. Another HCV core coding region antisense oligo, A446 (SEQ ID NO: 19), also showed weak inhibitory effects. Taken together, these results indicate that the sequence immediately downstream from the first AUG codon of the HCV core open reading frame is a good target sequence for antisense oligo inhibition of HCV RNA translation. A65 (SEQ ID NO: 2) also inhibited HCV RNA translation to a substantial degree, even though the region of HCV RNA that this oligo corresponds to is far upstream from the initiation codon. It is of interest that oligo A211 (SEQ ID NO: 8) binds to a putative 18S rRNA complementary sequence (Brown et al., Nucl. Acids Res. 20:5041-5045, 1992), and demonstrated no inhibitory effect for both type II and type III HCV RNA translation. The G-C contents and T_m s of the various oligos were not related to the degree of HCV RNA translation inhibition exhibited by the oligos (Table I).

A dose dependent inhibitory effect on HCV RNA translation was observed using A161 (SEQ ID NO: 6; Fig. 3). At the highest concentration of A161 used (molar ratio of oligo:RNA=100:1), HCV protein synthesis was almost completely blocked. It is noteworthy that even at this high concentration, the sense oligo (S120; SEQ ID NO: 32) had no inhibitory effect on HCV RNA translation. Furthermore, as a control, both of these oligos were incubated with an un-related mRNA encoding luciferase. Neither A161 (SEQ ID NO: 6) nor S120 (SEQ ID NO: 32) inhibited luciferase mRNA translation at any concentration employed (Fig. 3). Other antisense oligos actually were found to stimulate translation of luciferase mRNA (Table II). This observation is reminiscent of the observation that translation of HCV

- 19 -

RNA is stimulated by certain sense oligos (Table I). In summary, certain antisense oligos inhibit HCV RNA translation in a sequence specific manner *in vitro*, even though the same oligos were found to, if anything,
5 increase the level of translation of an unrelated mRNA.

To determine whether annealing of antisense oligos to target RNA prior to the *in vitro* translation reaction is required for effective inhibition of translation, translation reactions were carried out without the
10 annealing step. Antisense oligos were added just prior to the HCV RNA translation step, and the degree of inhibition was determined. In the absence of the annealing step, oligos showed the same, or higher, levels of inhibition of HCV RNA translation compared to
15 reactions carried out with the annealing step (Table II). **Specific inhibition of HCV RNA translation by short antisense oligos in the A377 (SEQ ID NO: 17) region**

Oligos A367 (SEQ ID NO: 16) and A377 (SEQ ID NO: 17) were the most effective among those tested as
20 inhibitors of HCV type II and type III RNA translation (Table I). Since there is a mutation at nucleotide position 350 in type III HCV RNA (Fig. 5), and because the sequence complementary to this position is contained within oligo A367, but not in A377, the HCV region
25 defined by oligo A377 (SEQ ID NO: 17) was selected for further study to determine the specific nucleotide sequence requirements for inhibition of HCV RNA translation. Different length oligos derived from the A377 (SEQ ID NO: 17)-defined region of HCV were tested
30 with type II HCV RNA in the *in vitro* translation assay. As nucleotides were deleted from the 3' end of the A377 (SEQ ID NO: 17) sequence, the degree of inhibition was reduced in proportion to the size of the deletion. In contrast, deletions from the 5' end of A377 (SEQ ID NO:
35 17) increased the inhibitory effect from 78.4% (A377; SEQ

- 20 -

ID NO: 17; 27 nucleotides) to 96.9% with A367 (SEQ ID NO: 16; 17 nucleotides). It is noteworthy that A362 (SEQ ID NO: 25), which is only 12 nucleotides in length, significantly inhibited HCV RNA translation (by 80.7%).

5 The inhibition of translation was substantially reduced using oligos of only 10 nucleotides in length, e.g., oligos A362 (SEQ ID NO: 27) and A360 (SEQ ID NO: 28). These experiments show that the HCV RNA region between nucleotide positions 351-367, which is highly conserved
10 in both type II and type III HCV clones, is an excellent viral RNA target for antisense oligo inhibition.

HCV cDNA synthesis with antisense oligos

Both the binding efficiencies and the nucleotide sequence specificities of the various antisense oligos
15 may play roles in determining the degree of inhibition of HCV RNA translation they exhibit. To determine the relative binding affinities of different antisense oligos to HCV RNA, cDNA synthesis assays were carried out. Oligos A65 (SEQ ID NO: 2) and A377 (SEQ ID NO: 17), which
20 inhibit HCV RNA translation, and oligos A103 (SEQ ID NO: 4) and A387 (SEQ ID NO: 18), which are derived from the same general regions of the HCV genome as oligos A65 (SEQ ID NO: 2) and A377 (SEQ ID NO: 17), respectively, but unlike the latter do not inhibit HCV translation, were
25 used in this analysis. The oligos were labeled with [γ -³³P]ATP, and the specific activities were normalized in all experiments. The same ratio of HCV RNA to oligo (1:10) used in the translation assays was used in the cDNA synthesis reactions. Fig. 5 shows the results of
30 these experiments. The predominant cDNA products fractionated at their expected sizes by urea-PAGE. Several smaller bands were detected in lanes 3 and 4 (Fig. 5), and were probably generated in part because of the secondary structure of the template RNA, which blocks
35 elongation of the cDNAs. The patterns of the DNA ladders

- 21 -

generated in the reaction containing oligo A377 (SEQ ID NO: 17; Fig. 5, lane 3), which inhibits HCV RNA translation, and the reaction containing oligo A387 (SEQ ID NO: 18; Fig. 5, lane 4), which does not inhibit HCV RNA translation, are nearly identical. These data show that inhibition of HCV RNA translation is not a function of non-specific binding of oligos to HCV RNA target sequences, but rather, is due to the specificity of the HCV RNA target sequence/antisense oligo interaction.

10 Further, these data show that the differences in inhibition of HCV RNA translation observed using different antisense oligos is not due to different binding affinities of the oligos, but rather is due to binding specificity of the oligos to HCV RNA, as the

15 levels of cDNA products produced in the reactions using different antisense oligos as primers are similar.

HCV RNA stability

RNase H activity has been proposed to be one of the major mechanisms for the inhibitory effects of antisense oligos (Uhlmann et al., Chem. Rev. 90(4):543-584, 1990). To determine the stability of HCV RNA during *in vitro* translation reactions, [³³P]-labeled HCV RNA was synthesized and purified by gel electrophoresis. Labeled HCV RNA was hybridized with oligos, and incubated with

25 rabbit reticulocyte lysates in the translation assay. After 15 minutes of incubation at 30°C, HCV RNA was extracted, and loaded onto gels under denaturing conditions. HCV RNA incubated without any oligo showed the expected full size transcripts, and minor degradation

30 products (Fig 6, lanes 4 and 5). However, RNAs hybridized with oligos demonstrated two additional major bands (Fig. 6, lanes 1 and 2). The sizes of the shorter RNA bands are consistent with their corresponding to cleaved products generated by RNase H activity. It is

35 noteworthy that labeled HCV RNAs were not completely

- 22 -

degraded under these conditions. Furthermore, similar levels of HCV RNA cleavage were observed for both inhibitory and non-inhibitory oligos. Thus, it is not likely that RNase H degradation is the major mechanism
5 whereby the above-described antisense oligos inhibit HCV RNA translation.

Other embodiments

From the above description, one skilled in the art can easily ascertain the essential characteristics of the
10 present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. It is understood that oligos having minor variations from the disclosed sequences
15 which do not affect the biological function (i.e., the ability of the oligo to hybridize to, and to inhibit the translation of, HCV RNA) of the oligos are within the invention.

All publications cited herein are fully
20 incorporated by reference herein in their entirety.

- 23 -

Table I. Percent Inhibition of Type II and III HCV**Translation by oligos**

					<i>Inhibition of protein synthesis^b (%)</i>	
		<i>Oligos^a</i>	<i>Size (nt)</i>	<i>GC (%)</i>	<i>Type II</i>	<i>Type III</i>
SEQ ID NO:	1	A37	28	57	5	ND
SEQ ID NO:	2	A65	28	54	74	54
SEQ ID NO:	3	A81	28	46	40	16 (1) ^c
SEQ ID NO:	4	A103	28	50	-1	-1 (1)
SEQ ID NO:	5	A147	28	71	20	14
SEQ ID NO:	6	A161	28	61	81	76
SEQ ID NO:	7	A175	28	57	55	88
SEQ ID NO:	8	A211	28	61	4	-20
SEQ ID NO:	9	A242	28	68	34	ND
SEQ ID NO:	10	A270	28	57	44	ND
SEQ ID NO:	11	A298	28	54	40	35
SEQ ID NO:	12	A312	28	57	45	18
SEQ ID NO:	13	A326	28	71	64	30
SEQ ID NO:	14	A339	27	70	53	50
SEQ ID NO:	15	A349	28	61	41	39 (1)
SEQ ID NO:	16	A367	27	44	98	95 (1)
SEQ ID NO:	17	A377	27	30	80	94
SEQ ID NO:	18	A387	27	37	15	22 (2)
SEQ ID NO:	19	A446	27	52	13	4 (3)
SEQ ID NO:	29	S38	28	54	-4	ND
SEQ ID NO:	30	S54	28	46	0	ND
SEQ ID NO:	31	S76	28	50	2	-15
SEQ ID NO:	32	S120	28	71	-21	-2
SEQ ID NO:	33	S148	28	57	-11	29
SEQ ID NO:	34	S271	28	54	-30	-33
SEQ ID NO:	35	S291	28	68	-37	-33
SEQ ID NO:	36	S312	28	68	-35	-25

^aA = Antisense oligo, S=sense oligo. The number after A or S indicates the position of the 5' end of the oligo. The underlining indicates oligos that inhibit HCV type II and III translation by greater than 50%.

^bInhibition assays were performed as described in materials and methods. Each oligo was used at 10 fold molar excess relative to HCV RNA. ND=not determined.

^cThe numbers in parentheses indicate the mutations in the target sequence of the type III clone.

- 24 -

Table II. Inhibition of HCV and Luciferase RNA translation by antisense oligos, and effect of the annealing step on the inhibition of HCV RNA translation

		<u>Inhibition of translation (%)</u>		Luciferase ^a
		annealing(+)	annealing(-)	
SEQ ID NO: 2	A65	75	73	-2
SEQ ID NO: 6	A161	77	83	-27
SEQ ID NO: 14	A339	60	63	-84
SEQ ID NO: 17	A377	84	93	-49

^a All in vitro translations with Luciferase RNA were performed with annealing steps

- 25 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Wakita, Takaji
Wands, Jack
- (ii) TITLE OF INVENTION: ANTISENSE INHIBITION OF
HEPATITIS C VIRUS
- (iii) NUMBER OF SEQUENCES: 38
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/240,382
(B) FILING DATE: 10 May 1994
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Clark, Paul T.
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NUMBER: 00786/221001
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

- 26 -

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATCTATGGTG GAGTGTCGCC CCCAATCG 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAGACAGTA GTTCCTCACA GGGGAGTG 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TAGACGCTTT CTGCGTGAAG ACAGTAGT 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

- 27 -

ACACTCATAC TAACGCCATG GCTAGACG 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACCACTATGG CTCTCCCGGG AGGGGGGG 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CACCGGTTCC GCAGACCACT ATGGCTCT 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATTCGGTG TACTCACCGG TTCCGCAG 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid

- 28 -

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCGGGTTGAT CCAAGAAAGG ACCCGGTC 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCGGGGGCAC GCCCAAATCT CCAGGCAT 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GACCCAACAC TACTCGGCTA GCAGTCTC 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TATCAGGCAG TACCACAAGG CCTTTCGC 28

- 29 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:**(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACTCGCAAGC ACCCTATCAG GCAGTACC 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:**(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGACCTCCCG GGGCACTCGC AAGCACCC 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:**(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGCACGGTCT ACGAGACCTC CCGGGGCA 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:**(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

- 30 -

GTGCTCATGG TGCACGGTCT ACGAGACC

28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	27
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTTTGAGGTT TAGGATTCGT GCTCATG

27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	27
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTTGTTT TTTGAGGTT TAGGATT

27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	27
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TGGTGTTACG TTTGGTTTTT CTTTGAG

27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 19:

(i) SEQUENCE CHARACTERISTICS:

- 31 -

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GTAAACTCCA CCAACGATCT GACCACC 27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TTTGGTTTTT CTTTGAGGTT TA 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TTTGGTTTTT CTTTGAG 17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TTTGGTTTTT CT 12

- 32 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TTTTTCTTTG AGGTTTAGGA TT 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	17
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CTTTGAGGTT TAGGATT 17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	12
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AGGTTTAGGA TT 12

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	12
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

- 33 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CTTTGAGGTT TA

12

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	10
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

AGGTTTAGGA

10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	10
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTTTAGGATT

10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CACTCCCCTG TGAGGAACTA CTGTCTTC

28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 30:

(i) SEQUENCE CHARACTERISTICS:

- 34 -

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

ACTACTGTCT TCACGCAGAA AGCGTCTA 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CGTCTAGCCA TGGCGTTAGT ATGAGTGT 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CCCCCCTCC CGGGAGAGCC ATAGTGGT 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

- 35 -

CTGCGGAACC GGTGAGTACA CCGGAATT

28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GCGAAAGGCC TTGTGGTACT GCCTGATA

28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GCCTGATAGG GTGCTTGCGA GTGCCCCG

28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TGCCCCGGGA GGTCTCGTAG ACCGTGCA

28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	29
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single

- 36 -

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CGAAUCCUAA ACCUCAAGA AAAACCAAA 29

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	29
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CAAUCCUAA ACCUCAAGA AAAACCAAA 29

What is claimed is:

- 37 -

1. A method of inhibiting translation of Hepatitis C Virus RNA, said method comprising contacting said RNA with an oligonucleotide substantially complementary to a portion of said RNA, said
5 oligonucleotide being an RNA or a DNA molecule comprising a sequence selected from the group consisting of SEQ ID NOS: 1-28, said oligonucleotide being between about 10 and about 400 nucleotides in length.
2. The method of claim 1, wherein said
10 oligonucleotide is less than about 250 nucleotides in length.
3. The method of claim 2, wherein said oligonucleotide is less than about 100 nucleotides in length.
- 15 4. The method of claim 3, wherein said oligonucleotide is less than about 50 nucleotides in length.
5. The method of claim 4, wherein said oligonucleotide is between about 12 and about 28
20 nucleotides in length.
6. The method of claim 1, wherein said Hepatitis C Virus is selected from the group consisting of Hepatitis C Virus types I, II, III, IV, and V.
7. The method of claim 1, wherein said Hepatitis
25 C Virus RNA is in an animal cell.
8. The method of claim 7, wherein said animal cell is an hepatocyte.

- 38 -

9. The method of claim 7, wherein said animal cell is in a mammal.

10. The method of claim 9, wherein said mammal is a human.

5 11. The method of claim 7, wherein said contacting is carried out by introducing into said animal cell a vector containing a sequence which is transcribed in said animal cell to produce said oligonucleotide as an oligoribonucleotide.

10 12. The method of claim 1, wherein said oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NOS: 2, 6, 7, 14, 16, 17, 20, and 23-26.

15 13. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 2.

14. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 6.

20 15. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 7.

25 16. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 14.

- 39 -

17. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 16.

18. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 17.

19. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 20.

20. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 23.

21. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 24.

22. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 25.

23. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 26.

24. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 8-13, 15, 18, 19, 21, 22, 27, and 28.

25. An oligonucleotide substantially complementary to a portion of Hepatitis C Virus RNA, said

- 40 -

oligonucleotide being an RNA or a DNA molecule comprising a sequence selected from the group consisting of SEQ ID NOs: 1-28, said oligonucleotide being between about 10 and about 400 nucleotides in length.

5 26. The oligonucleotide of claim 25, wherein said oligonucleotide is less than about 250 nucleotides in length.

 27. The oligonucleotide of claim 26, wherein said oligonucleotide is less than about 100 nucleotides in
10 length.

 28. The oligonucleotide of claim 27, wherein said oligonucleotide is less than about 50 nucleotides in length.

 29. The oligonucleotide of claim 28, wherein said
15 oligonucleotide is between about 12 and about 28 nucleotides in length.

 30. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide comprises a nucleotide sequence selected from the group consisting of
20 SEQ ID NOs: 2, 6, 7, 14, 16, 17, 20, and 23-26.

 31. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 2.

 32. The oligonucleotide of claim 25, wherein the
25 nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 6.

- 41 -

33. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 7.

5 34. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 14.

35. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 16.

10 36. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 17.

15 37. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 20.

38. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 23.

20 39. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 24.

40. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 25.

25 41. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 26.

- 42 -

42. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 8-13, 15, 18, 19, 21, 22, 27, and 28.

43. A vector comprising a nucleotide sequence which is transcribed in an animal cell to generate the oligonucleotide of claim 23, said oligonucleotide being an oligoribonucleotide.

44. The vector of claim 43, wherein said transcribed nucleotide sequence is operably linked to transcription control sequences that function in hepatocytes.

45. The oligonucleotide of claim 25, wherein said oligonucleotide is an oligodeoxynucleotide.

46. The oligonucleotide of claim 25, wherein said oligonucleotide is an oligoribonucleotide.

47. The oligonucleotide of claim 25, wherein said oligonucleotide is a phosphorothioate oligonucleotide.

48. The oligonucleotide of claim 25, wherein said oligonucleotide is a methylphosphonate oligonucleotide.

49. A therapeutic composition comprising the oligonucleotide of claim 25 in a pharmaceutically acceptable carrier.

50. A therapeutic composition comprising the vector of claim 43 in a pharmaceutically acceptable carrier.

1/5

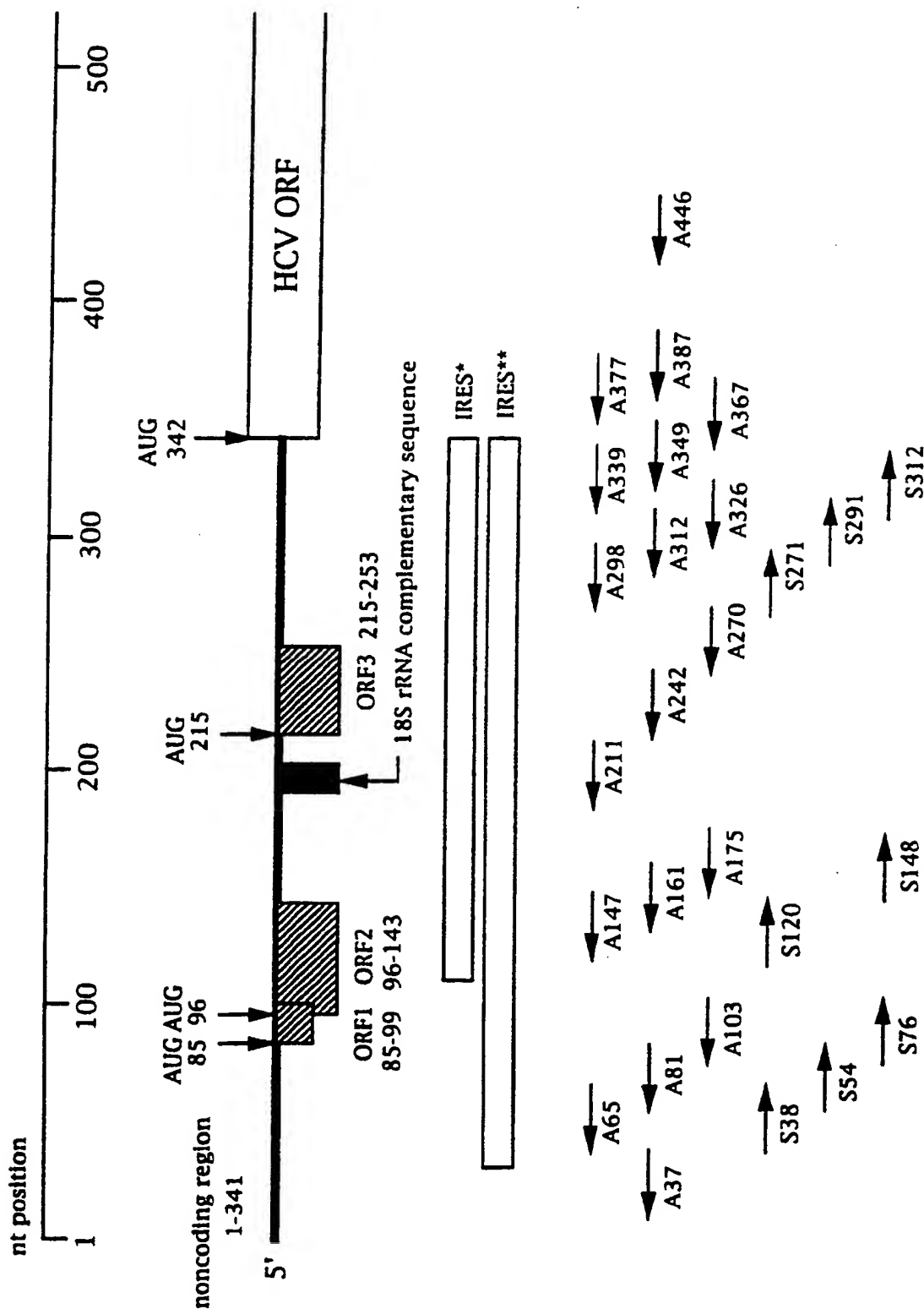
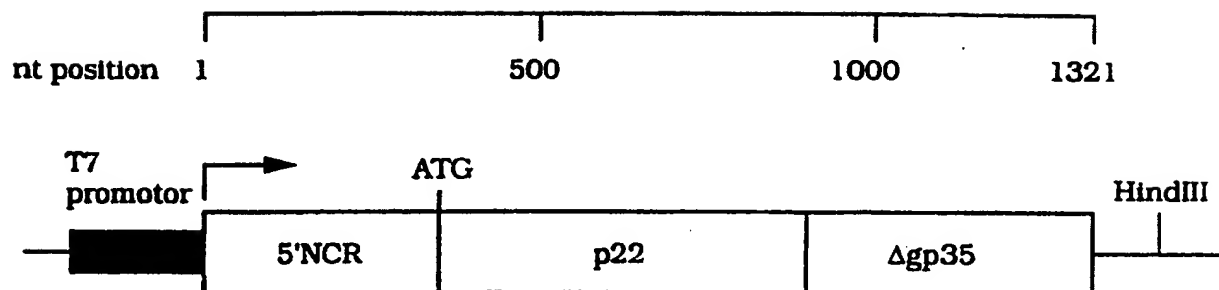


FIG. 1

2 / 5

**FIG. 2A**

3 / 5

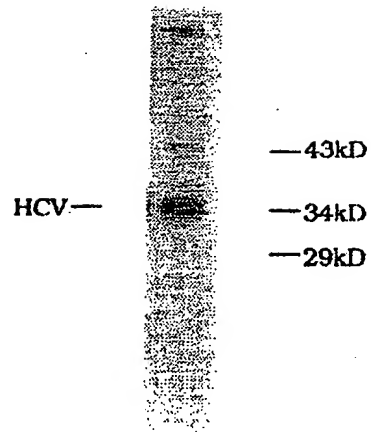


FIG. 2B

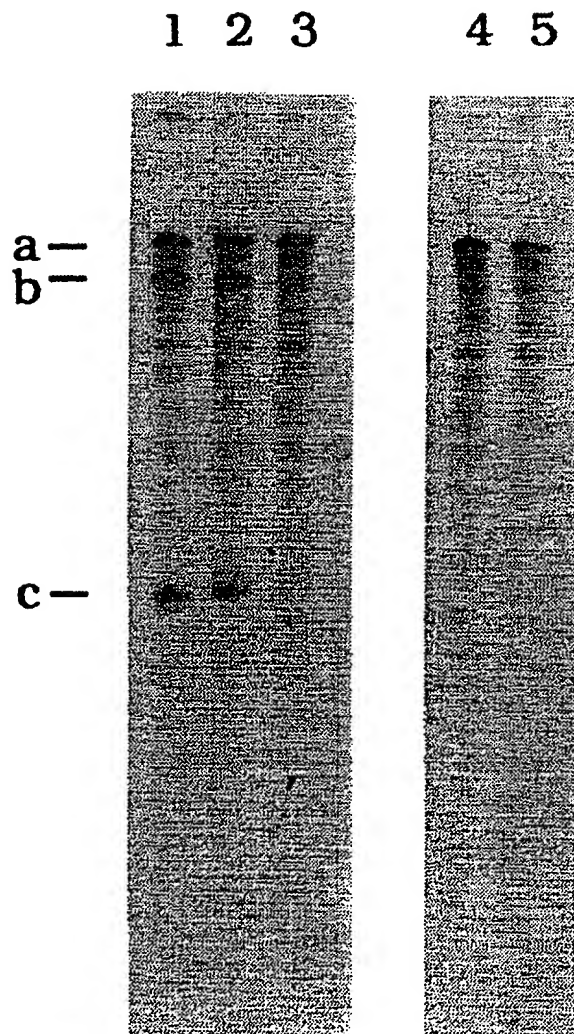
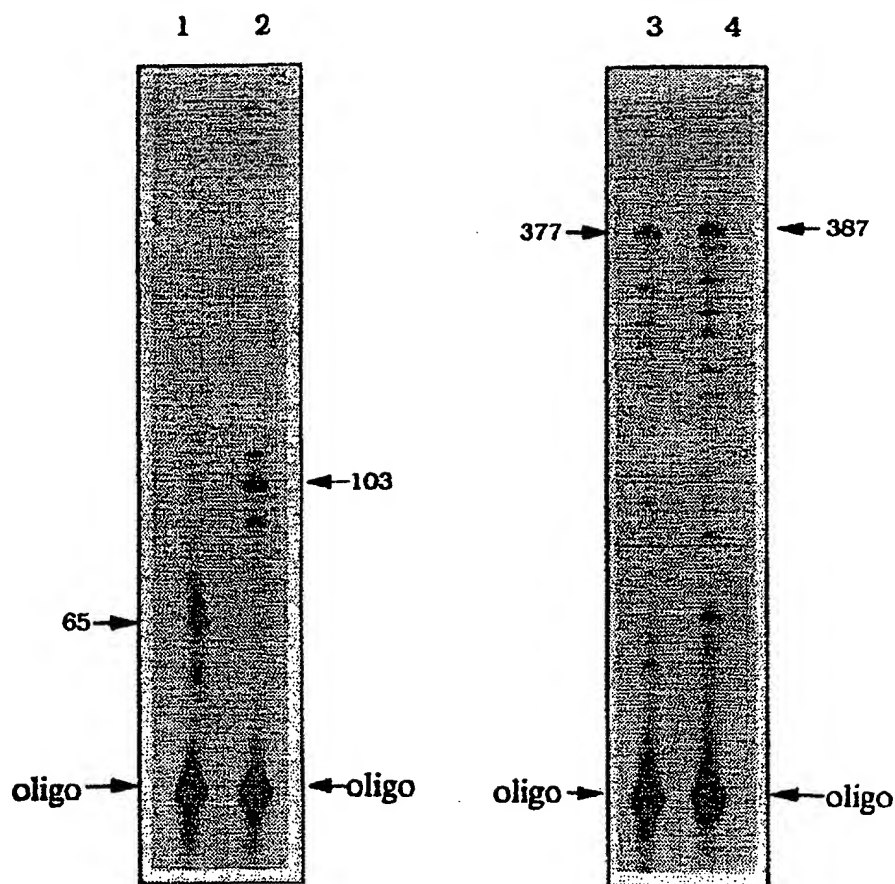


FIG. 6

4 / 5

**FIG. 5****HCV translation**

oligo	(-)	A161			S120	
oligo/RNA	0	1	10	100	10	100
HCV —						
Inhibition of protein synthesis(%)	0	18	74	93	-22	-13

FIG. 3A**Luciferase translation**

oligo	(-)	A161			S120	
oligo/RNA	0	1	10	100	10	100
Luciferase						
Inhibition of protein synthesis(%)	0	-7	-21	-1	-17	-46

FIG. 3B

Target HCV RNA sequence (nt 349-377)

TH 5' -CGAAUCCUAAACCUCUCAAAGAAAACCAA -3' (sense; SEQ ID NO:37)
 NT -A----- (sense; SEQ ID NO:38)

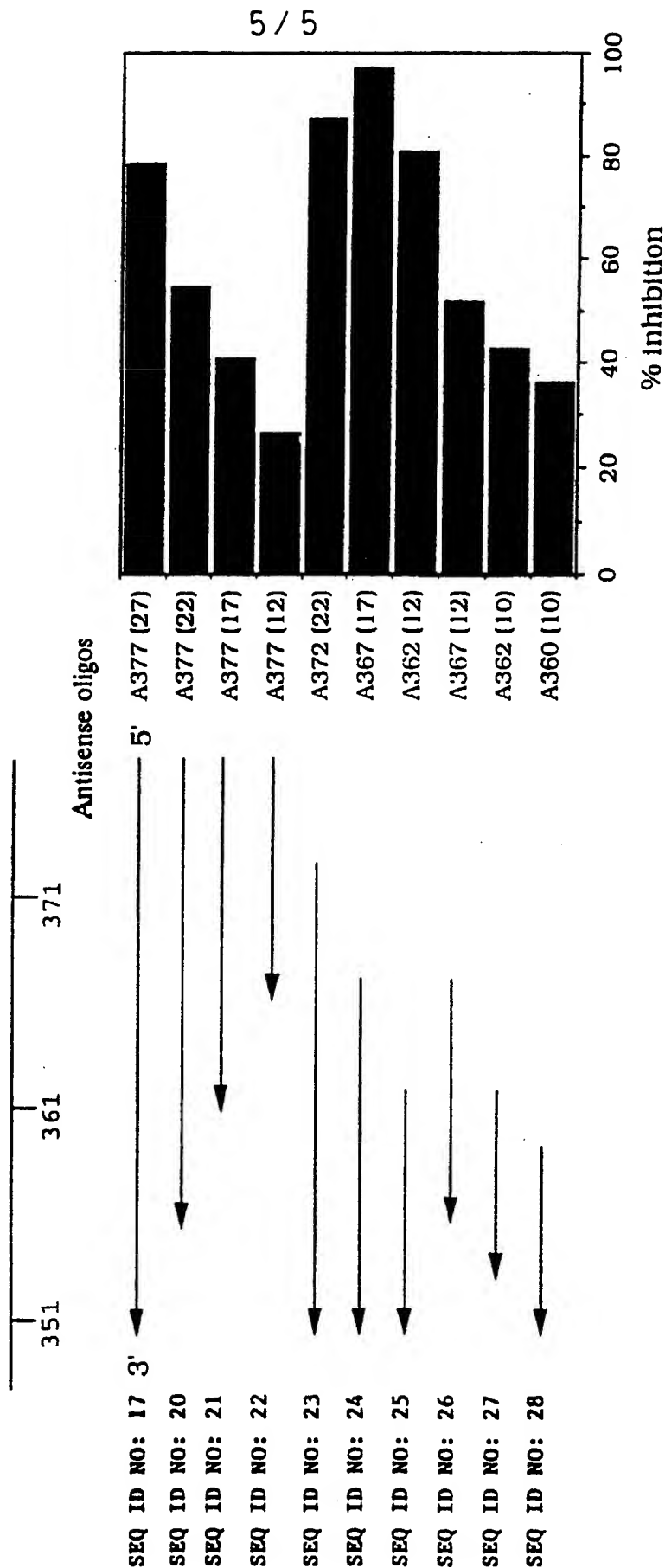


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05812

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/11, 15/51; A61K 48/00

US CL :514/44; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline

hepatitis, hepatitis C, oligonucleotide, oligodeoxynucleotide, antisense, antiviral

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Medicinal Chemistry, Volume 36, Number 14, issued 09 July 1993, Milligan et al., "Current Concepts in Antisense Drug Design", pages 1923-1937, see entire document.	1-50
Y	Science, Volume 261, issued 20 August 1993, Stein et al., "Antisense Oligonucleotides as Therapeutic Agents-Is the Bullet Really Magical?", pages 1004-1011, see entire document.	1-50
Y	Journal of Biological Chemistry, Volume 267, Number 18, issued 25 June 1992, Wu et al., "Specific Inhibition of Hepatitis B Viral Gene Expression <i>in Vitro</i> by Targeted Antisense Oligonucleotides", pages 12436-12439, see entire document.	1-50

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 JUNE 1995

Date of mailing of the international search report

10 JUL 1995

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MICHAEL J. NEWELL

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05812

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of General Virology, Volume 71, issued 1990, Goodarzi et al., "Antisense Oligodeoxyribonucleotides Inhibit the Expression of the Gene for Hepatitis B Virus Surface Antigen", pages 3021-3025, see entire document.	1-50
Y	EMBO Journal, Volume 12, Number 3, issued 1993, Offensperger et al., "In Vivo Inhibition of Duck Hepatitis B Virus Replication and Gene Expression by Phosphorothioate Modified Antisense Oligodeoxynucleotides", pages 1257-1262, see entire document.	1-50
T	Pharmacology and Therapeutics, Volume 65, issued 1995, Dusheiko, "Treatment and Prevention of Chronic Viral Hepatitis", pages 47-73, see entire document.	1-50
Y,P	US, A, 5,346,696 (KIM ET AL.) 13 September 1994, see entire document.	1-50
Y	Science, Volume 244, issued 21 April 1989, Choo et al. "Isolation of a cDNA Clone Derived from a Blood-Borne Non-A, Non-B Viral Hepatitis Genome", pages 359-362, see entire document.	1-50
Y	Advances in Experimental Medicine and Biology, Volume 342, issued 1993, Mizutani et al., "Inhibition of Mouse Hepatitis Virus Multiplication by Antisense Oligonucleotide, Antisense RNA, Sense RNA and Ribozyme", pages 129-135, see entire document.	1-50
Y,P	US, A, 5,372,928 (MIYAMURA ET AL.) 13 December 1994, see entire document.	1-50